

**WINTER SCHOOL  
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**Course Manual**



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# MARINE INVERTEBRATE TISSUE CULTURE TECHNIQUES AND ITS APPLICATION IN PEARL PRODUCTION

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## Introduction

Tissue culture, in general, is being carried out in various fields of medical and agricultural research. The study has been commenced since long back to understand the cell type, cell behaviour, cell structure, cell multiplication, cell reaction to drugs etc. It has become a vital tool in micro pathological and immunological studies aiming at finding solutions to certain diseases. Plant tissue culture has reached an advanced stage of achieving an entire plant from a single cell. All these studies are concerned with plants / animals which are related to freshwater species. Marine invertebrate tissue culture is not only a new origin but also a new field of research concerned with marine animals. Primary aim of the study is to formulate suitable culture media specific to each species and to establish cell lines. Later the study is extended to commercial aspects of producing *in-vitro* pearl from pearl oysters in Japan. Extensive works have been carried out on cell proliferation and its behaviour in a medium developed specifically for the species and to formulate suitable medium based on the results obtained. The countries like Japan, China, United States and Canada initiated marine invertebrate tissue culture. Among these countries, Japan is the pioneer country carrying out research in pearl oyster for the purpose producing *in-vitro* pearl through tissue culture. Visualizing the importance of the work in view of deterioration of natural environment India too entered in to the field of marine invertebrate tissue culture research, as it is one of the pearl producing countries in the world. Expertise in the field of research has already been developed and a fully functional marine invertebrate tissue culture laboratory has been established at Tuticorin for the first time in India. Cultures are organized since 1996.

## Set up of tissue culture laboratory

Generally the tissue culture laboratory should be compact with different modules so as to contain contamination by effectively maintaining high-grade hygienic conditions. It is fully air-conditioned. The entrance room is the one where the records are maintained and discussions are held prior to organization of cultures. Animal sterilization room is arranged on the left side of entrance room having U.V. sterilization unit and provisions for running seawater supply. The entrance room leads to preparation room where preparation of culture media, saline solutions, extracts, tissue culture materials etc., are carried out. The preparation room proceeds to dressing room and to operation room or clean room. A dark chamber or otherwise called 'Pass Box' is situated in between the preparation room, dressing room and clean room. It has three doors with a U.V. light on its top to keep the materials always sterile. The doors are arranged in such a way that one door is facing

preparation room through which sterile materials are placed inside, the other on the dressing room from where the dress materials are taken out and the third one on the operation room from where the materials are taken during organization of cultures.

### **Preparation of culture media and balanced salt solution**

The marine mollusc medium (MMM) is constituted based on the composition of haemolymph of each species. Refinement of medium is done periodically based on the results obtained in the cell culture. There are a few media developed for marine mollusc. They are Medium 199, P35, L-15 and Ham's F 12. The media are commercially available. They can either be prepared by following the formula.

The balanced salt solution (BSS) is prepared in the following manner.

Na. K. solution	125 ml
Mg. Solution	50 ml
Triple distilled water	200 ml
These are mixed, autoclaved and taken to clean room	
Ca. solution	50 ml
Triple distilled water	50 ml

These two solutions are mixed, autoclaved and taken to clean room. Mixing of the above two solutions and the following is done on the clean bench.

Glucose	5 ml
NaHCO <sub>3</sub>	5 ml
NaH <sub>2</sub> PO <sub>4</sub>	5 ml
Kanamycin	0.5 ml
Penicillin	5 ml
Fungizon	5 ml
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Total	500 ml
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### **Preparation of animals and tissues**

The test animals are depurated for a minimum period of 3 days in U.V.treated running seawater. The depurated animals are wiped with externally with 70 % alcohol and taken to clean room. The mantle tissues of test animals are excised and washed in BSS to get rid of mucus and other adhering particles. The tissues are cut in to tiny pieces of 1 square mm in size.

### **Culture techniques**

**Flask and Petri dish cultures:** Before introducing the fragments of tissue in to the culture flask the mouth of the flask is shown to isopropanol flame for sterilization. Tissues are placed inside the flask with the help of a needle. The tissues are allowed to stick on to the flask and 3 ml of medium is added. A similar inoculation is made in petri dishes also. The culture plates are placed in CO<sub>2</sub> incubator and maintained at 25-28°C.

**Cell well culture:** The cell well is otherwise called as micro plates. There are different types of cell wells. The size of 24 wells is 16 mm in diameter and 17 mm in height and the size of 96 wells is 6.4 mm diameter and 11 mm height. The cell well is provided with a cover. The cell well is used to culture single cell for the purpose of cloning. 3 to 4 drops of medium are added to each well. The cell wells are kept in CO<sub>2</sub> incubator at 25-28°C.

**Medium change:** Medium change is normally done on alternative days. Periodicity of medium change is determined by observing the condition of the cultures. Culture flasks are taken to clean bench after wiping with 70 % alcohol. When the flask is opened, it is shown to flame. Much care is taken during medium change. A separate pipette is used for each flask. Half of the medium is changed during first and second time and subsequently the whole medium is changed. At times cell suspension is centrifuged and fresh inoculations are made. In some established cell lines the cells are active and hence the entire medium is changed.

### **Organization of cultures**

- 1. Primary culture:** The processed tissue is treated with trypsin for the purpose releasing the cells from the tissue. To effect this the cut pieces of tissues are placed in trypsinisation flask containing 30 ml on marine mollusc calcium magnesium free phosphate buffer solution (MM CMF PBS) with 0.05 % trypsin. A teflon stirrer is used in the flask for proper dissociation of tissues and dispersion of cells. The stirring is done for 10-15 minutes at 1200 rpm. The cell suspension is first filtered through 150 µm sieve and then through 60 µm sieve. The filtrate is centrifuged at 4°C for 5 minutes at 800 rpm and the supernatant solution is removed gently without disturbing the precipitate. A drop of medium is added to the precipitate and mixed well. The mixture containing free cells is distributed to different flasks or petri dishes by means of Pasteur's pipette. 3 ml of medium is added to each flask and the flasks are placed in CO<sub>2</sub> incubator at 25-28°C.
- 2. Explant culture:** For explant culture of tissues, fragments of tissues are processed in balanced salt solution (BSS) and inoculated in the flasks or petri dishes. 3 ml of medium is added to each flask. The cells from the explant proliferate in large numbers and migrate away by adhering to the bottom of the flask. The round epithelial-like cells and fibroblast-like cells are seen in the cultures. The cells do multiply in *in-vitro* cultures and increase in numbers forming cell sheet. When a cell sheet is fully formed, it is due for subcultures or for cryopreservation of cells. At ideal conditions the cells develop pseudopodia and form a network to cover the entire surface of the flask as organic matrix. The migrated cells are stationed at places and formed pearl sac. The organic matrix induces the cells to secrete crystals.
- 3. Organ culture:** The processed fragments of tissues are placed on a raft in petri dishes. The raft may be at any form as per the requirement of the experiment. In organ culture the explant tissue is not immersed in the medium but it is kept in such a way that the medium is filled up to the lower phase of the tissue leaving the upper phase with air contact. In such case the cells are kept intact without dislodging their

positions. The interaction and integration of the cells perform their original functions of forming organic matrix and pearl sac. The cells secrete nacreous crystals and deposit on the matrix. As the mantle cells are responsible for the formation of shell, the cells secrete prismatic layer in hexagonal form. Each hexagonal segment is bordered by interlamellar organic matrix.

### **Preservation of cells**

Cells to be preserved by freezing would be released from the culture flask by adding 0.25 % of trypsin. The cell suspension with 3 to 6 ml of medium is centrifuged for five minutes at 1200 rpm at 4°C. Supernatant water is removed and 2 ml of medium and 2 ml of Minimum Essential Medium (MEM) with Dimethyl Sulfoxide (DMSO) 7.5 % mixture were added drop by drop. The 4 ml suspension is divided into four parts and kept in four freezing vials. After the vials are sealed and labeled, there are frozen at the rate of -1°C at every minute. Freezing is done at three stages, first at 0°C for 30 minutes, then at -20°C for 60 minutes and thirdly at -70°C for 6 months and finally at -196°C for one or two years in liquid nitrogen.

In order to protect from damages of cells during storage, DMSO 7.5 % and glycerine 10 % are used along with medium. Freezing of cells is done mainly for three reasons.

1. During cell line the cells may change their enzyme activity, chromosome number etc. Therefore it is essential to freeze these cells at a particular stage of cell line and then rejuvenated.
2. There may be contamination in cell line. To prevent this cells are frozen at periodic intervals.
3. In an established cell line the cells can be cultured to a maximum of 50 times. In some other cell line, cells are likely to die at any time. Such cell lines can be sub-cultured only for 30 times. Freezing of these cells may extend the period of cell line.

### **Application of tissue culture techniques**

There is an increasing use of tissue culture in various fields of biological research. Tissue culture techniques are being adopted in Marine Invertebrates since in recent years. By conducting tissue culture, valuable information could be collected on aspects of like cell structure, cell division, cytogenetics, cell physiology and cell viability. Tissue culture techniques are useful in studying the structural as well as functional aspects of cells, tissues or organs by culturing them *in-vitro*. The techniques are employed in investigating the effect of chemicals and radioactive elements on normal tissues and cancer cells and in microbiology, pathology and in the production of vaccines. Results obtained may help in finding out methods of curing several diseases. Careful studies in tissue culture will help in transplantation of tissues and cells among members of a species or from one species to another species. In recent years tissue culture technique is being used in the production of *in-vitro* pearls from pearl producing molluscs.